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Research Report

Profound hypothermia determines the anticonvulsant and neuroprotective effects of swim stress

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ABSTRACT

In contrast to most stressors that appear to be proconvulsant in nature, forced swimming (or swim stress) produces substantial anticonvulsant effects. Here we describe a series of experiments designed to identify the specific factors of swim stress (e.g., duration, swimming behavior, water temperature, and frequency of exposure) that are essential for the emergence of anticonvulsant effects in the rat. Our results revealed that the anticonvulsant effect of swim stress against lithium–pilocarpine convulsions occurred only when swim durations were at least 5 min in length and in water temperatures of 20 °C or less. Moreover, this anticonvulsant effect was not associated with habituation even after 10 days of repeated swimming. Treatment with lithium–pilocarpine coupled with 10 °C or 20 °C swim stress for 10 min caused pronounced hypothermia (10 to 15 °C reduction in body temperature) that required at least 12 h to return to baseline. One day after seizures were induced, swim stressed rats showed significantly fewer degenerating neurons in the hippocampus as revealed by Fluoro-Jade B staining. These results suggest that dramatically lowered body temperature could be the critical factor that produces the anticonvulsive and neuroprotective effects of swim stress.

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1. Introduction

Many epileptic patients maintain that physical and/or emotional stress can trigger the occurrence of seizures (da Silva et al., 2005; Frucht et al., 2000; Haut et al., 2003; Neufeld et al., 1994). Although most clinicians accept the association between stress and the precipitation of seizures, little empirical evidence for such a link exists (cf. Lai and Trimble, 1997). Complicating matters further, stress can afford protective or anticonvulsant effects against seizures for some epileptic patients (Kemph and Feldman, 1968; Neugebauer et al., 1994; Zegans et al., 1964). Therefore, identifying potential factors that affect the onset of seizures, even by a few minutes, can have important clinical implications because seizure control is not achieved in over 30% of epileptic patients (Aird, 1983).

In experimental animals, proconvulsive and anticonvulsive effects of stress have also been reported and it appears

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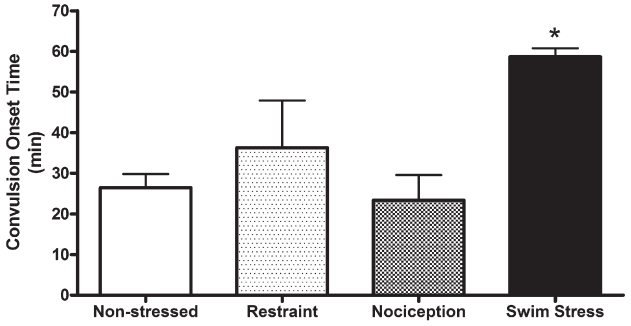

Fig. 1 – The mean convulsion onset time for rats that were exposed to different acute stress conditions before administration of pilocarpine. Results are expressed as mean ± S.D. *P < 0.001 differs from non-stressed controls.

that these effects are highly dependent on the type of stressor and the convulsant used to generate seizures (De Lima and Rae, 1991; Drugan et al., 1985; Rae et al., 1990). The forced swim stress test (Porsolt et al., 1977) has been extensively used to discover drugs with putative antidepressant properties and has become an increasingly important tool for understanding the neurocircuitry of the stress response (Connor et al., 1997; Cryan et al., 2005). Recently, we have shown (Galic et al., 2004), as well as others (Pericic et al., 2000), that a 10 min bout of swim stress at room temperature reduces the incidence and/or severity of behavioral seizures induced by a variety of convulsant drugs. In a set of comprehensive pharmacological studies, Pericic et al. identified several critical neurochemical systems that modulate the protective effect of swim stress on seizure threshold by showing that this stressor might engage an endogenous anticonvulsant circuit that mitigates the propagation and intensification of epileptiform events (Pericic et al., 2001a, 2001b, 2007).

Although forcing rats to swim in a confined space from which they cannot escape is undoubtedly anxiogenic and psychologically stressful to the animal (Cryan et al., 2005; Dayas et al., 2001; Porsolt et al., 1977), swimming for extensive periods (often 10–15 min or longer) in relatively cold water (normally 20 °C or lower) is also a physical stressor that results in profound changes in physiological homeostasis, such as increased motor activity, cardiovascular changes, and marked decreases in body temperature (Linthorst et al., 2008; Porsolt et al., 1979; Stone, 1970). While many neurochemical systems that are altered following swim stress (see de Groote and Linthorst, 2007; Engelmann et al., 2002, 2006; Linthorst and Reul, 2008; Wotjak et al., 1998, 2001) could serve as likely candidates in attenuating seizure activity, we considered the possibility that hypothermia might play a significant role in mediating the anticonvulsant effect of this stressor. Because hypothermia can influence several aspects of neural functioning, including the kinetics of ion channels (Rosen, 1996, 2001), presynaptic release (Busto et al., 1987), and/or the amplitude and duration of inhibitory after-hyperpolarization (Zarrindast and Oveissi, 1988), these temperature-dependent changes in neuronal functioning may have profound effects on the generation of seizures and overall epileptic activity. Our results clearly show that swimming even in room temperature water (~20 °C) causes significant hypothermia and that the induction of hypothermia that accompanies swim stress may be a substantial factor underlying its anticonvulsant efficacy. These finding may have important consequences for the interpretation of many studies that utilize the swim stress paradigm in pharmacological and behavioral research.

2. Results

2.1. *Experiment 1: comparison of swim stress with other acute stressors on seizure susceptibility*

Fig. 1 shows the mean convulsion onset time for rats exposed to various stress conditions. There was a statistically significant difference in convulsion onset time among groups of rats exposed to the different stressors [$F(3,16)=26.80$, $P<0.001$]. *Post hoc* analyses showed that subjects that received 10 min of swim stress before treatment with pilocarpine had significantly longer convulsion onset times than non-stressed controls [$P<0.001$]. In contrast, the rats that received nociceptive stress or 4 h of restraint stress before treatment with pilocarpine did not have convulsion onset times that differed from non-stressed controls [all P's >0.095].

2.2. *Experiment 2: effect of swim duration on seizure susceptibility*

Fig. 2 shows the mean convulsion onset time for rats exposed to different durations of swim stress. Swim duration had a significant effect on convulsion onset time [$F(4,15)=21.13$, $P<0.001$]. *Post hoc* analyses showed that rats that swam for at least 5 min or longer had significantly longer convulsion onset times than non-stressed controls [all P's <0.001]. In contrast, there was no difference in convulsion onset times between non-stressed controls and rats that swam for 1 min [$P=0.38$].

2.3. *Experiment 3: effect of repeated presentations of swim stress on seizure susceptibility*

Fig. 3 shows the mean convulsion onset time for rats exposed to different numbers of swim sessions prior to treatment with

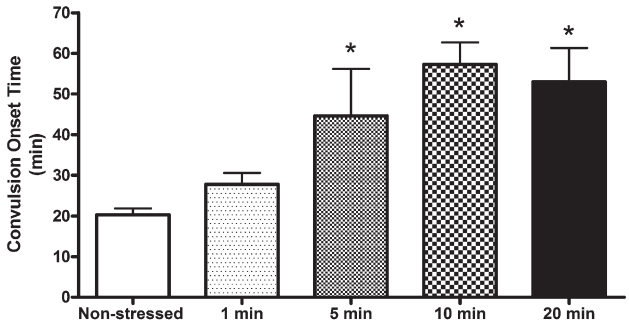

Fig. 2 – The mean convulsion onset time for non-stressed rats or rats exposed to 1 min, 5 min, 10 min, or 20 min of swim stress at 20 °C. Results are expressed as mean ± S.D. *P < 0.001 differs from non-stressed controls.

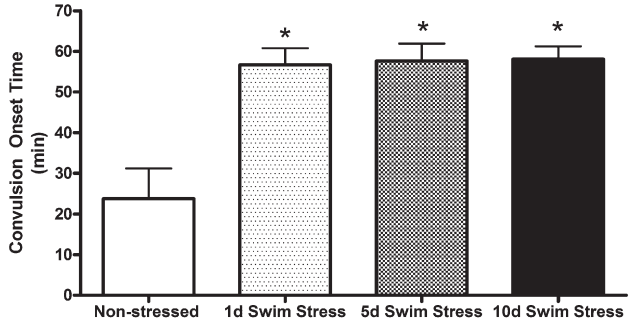

Fig. 3 – The mean convulsion onset time for non-stressed rats or rats that received swim stress for 10 min at 20 °C for 1 day, 5 days, or 10 days. Results are expressed as mean ± S.D. *$P<0.001$ differs from non-stressed controls.

pilocarpine. Despite repeated presentations of the stressor, all rats exposed to swim stress had significantly longer convulsion onset times than non-stressed rats [$F(3,16)=56.29$, $P<0.001$]. Importantly, we did not find significant differences between rats that received one session of swim stress and those that received 5 or 10 days of daily presentations of swim stress [all P's>0.62].

2.4. *Experiment 4: effect of swim behavior on seizure susceptibility*

Table 1 provides the mean convulsion onset time and duration of swimming behaviors for the rats exposed to the various swimming conditions prior to the administration of pilocarpine. Rats that swam in either the restricted or large open swim environments had significantly longer convulsion onset times than non-stressed controls [$F(2,15)=17.90$, $P<0.001$]. Interestingly, this effect persisted despite the fact that rats exposed to the large open swim environment engaged in different durations of immobility [$P<0.003$], swimming [$P<0.001$], and struggling [$P<0.003$] compared to rats exposed to the restricted swim environment. In addition, there was no significant correlation between convulsion onset time and swimming [$r=0.16$], immobility [$r=-0.15$], or struggling [$r=-0.12$] behaviors [all P's>0.62].

2.5. *Experiment 5: effect of water temperature on seizure susceptibility*

Fig. 4A shows the mean convulsion onset time for the rats exposed to different water temperatures during swimming. Water temperature had a significant effect on convulsion onset time [$F(3,13)=43.10$, $P<0.001$]. *Post hoc* analyses revealed that rats that swam at 20 °C or 10 °C had significantly longer convulsion latencies than non-stressed controls [all P's<0.001]. In contrast, rats that swam at 35 °C were not significantly different from non-stressed controls [$P=0.90$].

Fig. 4B shows the mean core body temperature for rats that swam at each temperature prior to treatment with pilocarpine. As expected, there were no differences in baseline body temperatures among the groups [all P's>0.80]. However, following the administration of pilocarpine, there were significant main effects for time [$F(7,105)=36.20$, $P<0.001$] and water temperature [$F(3,15)=22.12$, $P<0.001$], and a significant interaction between time and water temperature [$F(21,105)=8.20$, $P<0.001$]. For non-stressed controls, administration of pilocarpine produced mild (~2 °C) hyperthermia that was noticeable by 30 min after treatment and persisted for at least 3 h before returning to baseline levels. In contrast, swim stress resulted in the induction of severe hypothermia that was dependent on the temperature of the water (i.e., 10 °C > 20 °C > 35 °C). After treatment with pilocarpine, this pronounced hypothermic effect persisted for at least 12 h before returning to baseline for the rats that swam at 20 °C and 10 °C. In contrast, swimming at 35 °C resulted in an initial decrease in body temperature; however, these changes to body temperature were no longer statistically different from non-stressed controls after ~6 h [$P=0.16$] [Fig. 4B]. Finally, it is worth mentioning that control rats forced to swim at the same temperatures (10 °C, 20 °C, or 35 °C, 3 rats per condition) but not treated with pilocarpine also showed an initial decrease in body temperature that returned to baseline temperature by ~2 h after termination of the stressor.

2.6. *Experiment 6: effect of swim stress on seizure-induced neuronal degeneration*

Consistent with findings presented above, rats subjected to swim stress in room temperature water had significantly longer convulsion onset times than non-stressed controls [58.2 ± 2.2 min vs. 26.4 ± 3.6 min, $P<0.001$]. The effect of swim stress on the neuronal degeneration produced by lithium–pilocarpine convulsions that were allowed to continue for 30 min is shown in Fig. 5. Twenty-four hours after lithium–pilocarpine seizures were induced, swim stress rats showed a clear reduction in the number of Fluoro-Jade B positive cells throughout the hippocampal regions examined [Fig. 5A]. Although a few scattered degenerating cells could be detected, they were localized mainly to the CA3 pyramidal layer and

Table 1 – The mean (±S.D.) convulsion onset time and the total percentage of time spent immobile, swimming, and struggling during a 10 min exposure of swim stress at 20 °C in a restricted or large environment

	Convulsion onset time (min)	% Time spent immobile	% Time spent swimming	% Time spent struggling
Control	26.6 (±3.7)[a]	–	–	–
Large environment	54.6 (±7.0)[b]	8.3 (±4.4)[i]	72.8 (±5.3)[i]	18.8 (±5.6)[i]
Restricted environment	48.7 (±12.4)[b]	19.6 (±5.7)[ii]	47.3 (±5.3)[ii]	33.0 (±7.0)[ii]

[a] vs. [b] ($P<0.05$; Dunnett's two-tailed), [i] vs. [ii] ($P<0.003$; two-tailed *t*-test).

sometimes in the polymorphic layer of the dentate gyrus for some animals [Fig. 5A]. These cells were always fewer in number than what was found in non-stressed rats that experienced seizures of a similar length [$P<0.001$, Fig. 5B]. In contrast, the non-stressed rats had a large number of Fluoro-Jade B labeled cells in the hippocampus [Fig. 5A], located mainly within the stratum pyramidale layer of CA1 and CA3b (the curved portion of CA3), and the polymorphic layer of the dentate gyrus. All labeled cells fluoresced brightly green and had morphological characteristics suggestive of degenerating

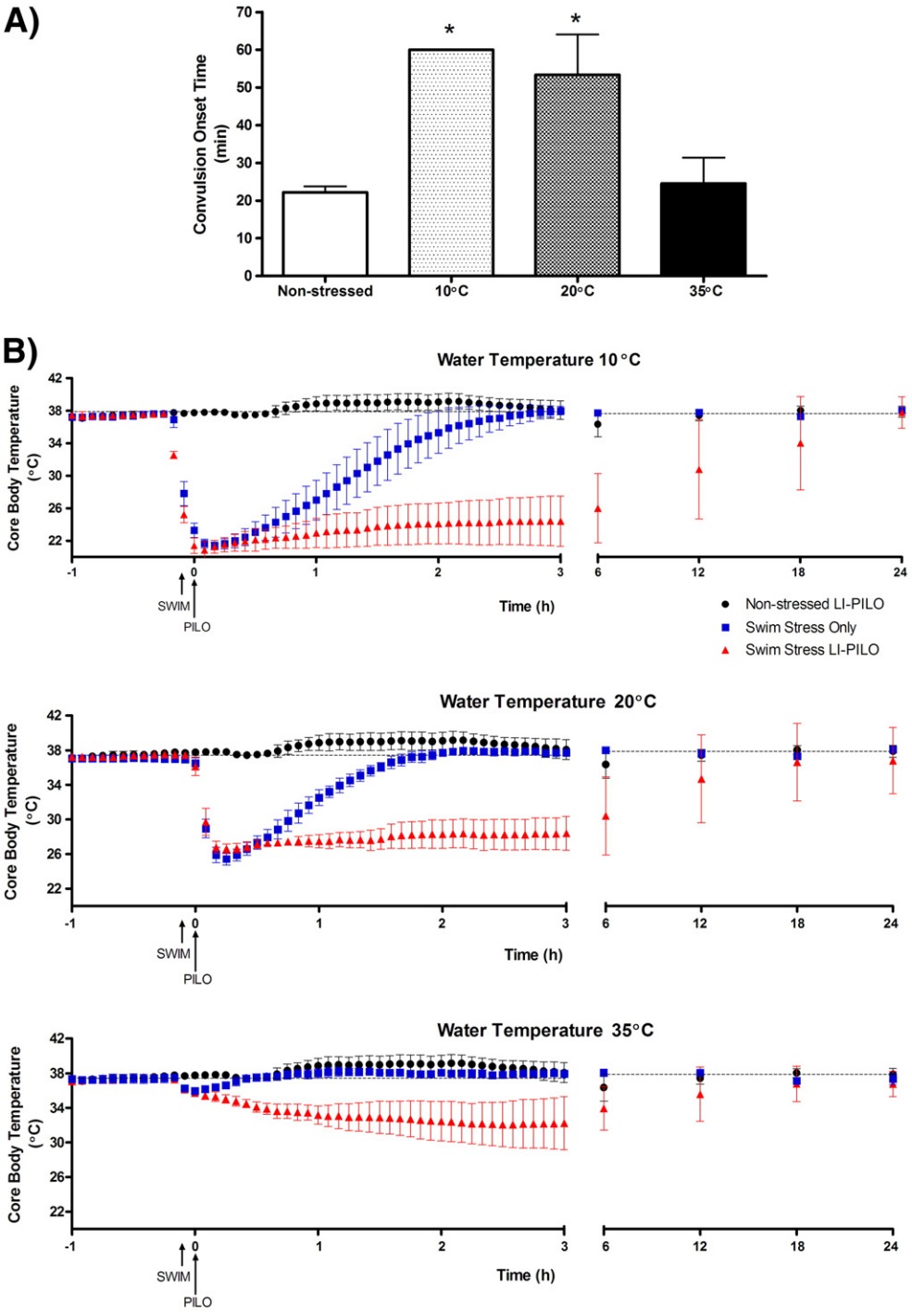

Fig. 4 – Effect of different water temperatures during swim stress on susceptibility to lithium–pilocarpine convulsions. (A) The mean convulsion onset time for non-stressed rats or rats that swam at 10 °C, 20 °C, or 35 °C water temperatures. Results are expressed as mean ± S.D. *$P<0.001$ differs from non-stressed controls. (B) The mean ± S.D. core body temperature changes over a 24 h period following administration of pilocarpine for non-stressed rats or rats that swam at 10 °C, 20 °C, or 35 °C water temperatures. For the purposes of comparison, the same non-stressed rats are re-plotted for each of the water temperatures. A separate group of rats that received 10 min of swim stress but not administered pilocarpine (Swim Stress Only) are also included. Dotted line indicates mean baseline core body temperature for 1 h prior to administration of pilocarpine.

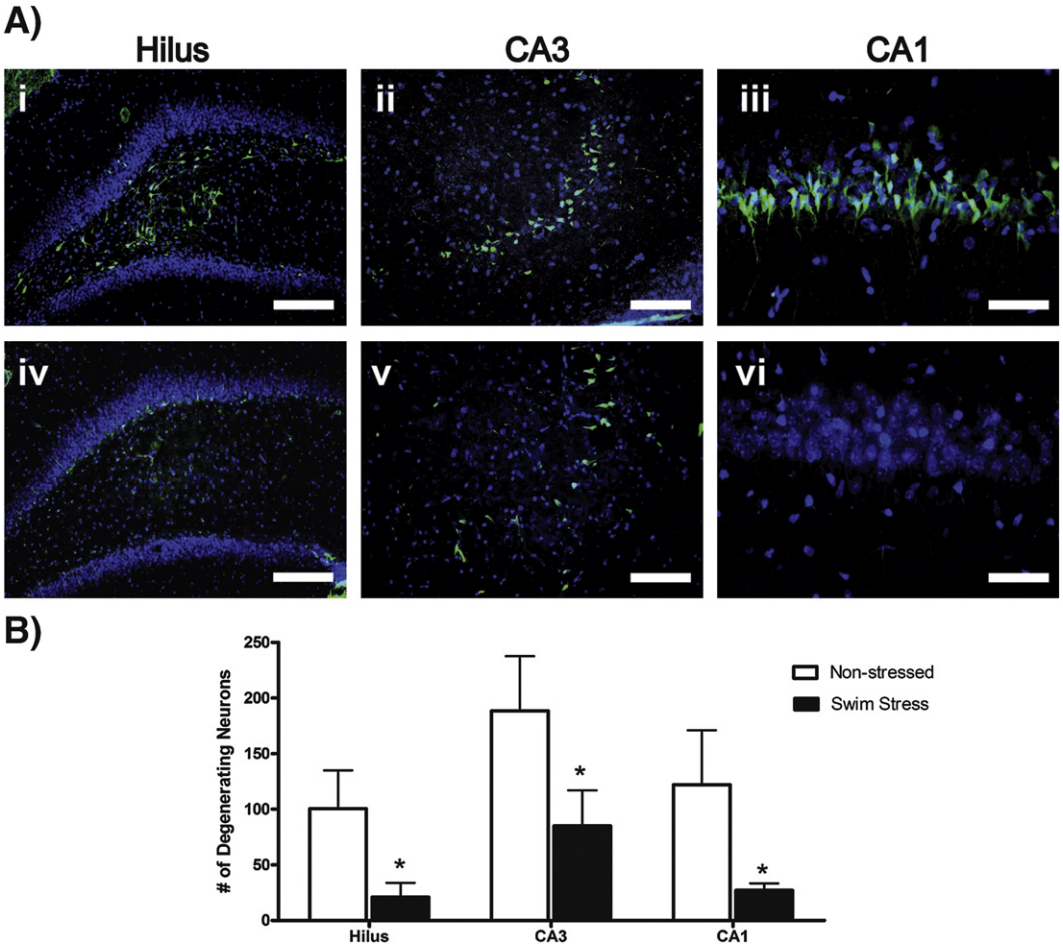

Fig. 5 – Effect of swim stress on neurodegeneration 24 h after lithium–pilocarpine convulsions. (A) Representative photomicrographs of Fluoro-Jade B staining in the hippocampus of non-stressed or swim stressed rats. i, ii, iii: In non-stressed rats, there was an increase in the number of degenerating neurons primarily within the stratum pyramidale of CA1 and CA3, as well as in the polymorphic zone of the dentate gyrus 24 h after seizure. iv, v, vi: In swim stressed rats, a few scattered degenerating neurons can be seen in the CA3 pyramidal layer 24 h after seizure. There is an absence of degenerating neurons in the CA1 pyramidal layer. Scale Bar: 200 μm (i,iv), 100 μm (ii,v), 50 μm (iii,vi). Sections are counterstained with Hoechst 33342. (B) The total number of degenerating neurons in the various hippocampal regions 24 h after lithium–pilocarpine convulsions. Results are expressed as mean ± S.D. *P < 0.01 differs from non-stressed controls.

neurons, such as shrunken cell bodies and pyknotic nuclei (Fujikawa, 1996). It should be noted that 2 non-stressed rats died during the 24 h period after treatment with pilocarpine despite administration of diazepam and as a result their data could not be included in the histological assessment.

3. Discussion

The majority of clinical and experimental evidence suggests that stress can facilitate the occurrence of seizures in many epileptic patients. However, the specific case of swim stress, which provides protection against seizures, may be an important exception to this rule. We have now identified specific elements associated with swim stress that could explain why this particular form of stress exerts protective effects, whereas the vast majority of psychological and physical stressors appear to facilitate seizures. First, the effect of swim stress is dependent on the duration of exposure because swim times of at least 5 min are necessary for protection against lithium–pilocarpine convulsions. Second, repeated exposures to swim stress do not reduce the anticonvulsant effect of this stressor. Third, the effect of swim stress appears to be highly dependent on the temperature of the water because temperatures of 20 °C or less resulted in persistent hypothermia that provided protection against the neurodegenerative effects of lithium–pilocarpine convulsions. These results suggest that swim stress can produce both anticonvulsive and neuroprotective effects through its action on body temperature.

3.1. *Effect of different forms of stress on lithium–pilocarpine convulsions*

Recent data suggests that different types of stress may not necessarily elicit comparable molecular, behavioral, and/or

physiological changes (see Bowers et al., 2008; Gregus et al., 2005; Pacak and Palkovits, 2001; Reyes et al., 2003), and consequently, they may have differential effects on seizure susceptibility. In contrast to the profound anticonvulsant effect of swim stress on convulsion onset time, we found that stressors such as restraint or thermal nociception were largely ineffective at delaying lithium–pilocarpine convulsions. Although our results do not preclude the possibility that more intense stressors (e.g., De Lima and Rae, 1991; Oliverio et al., 1983; Persinger et al., 2002) might be as effective as swim stress in decreasing seizure susceptibility, they do argue that the anticonvulsant effect of swim stress cannot be simply explained by anxiogenic or fear-related factors associated with swimming for survival. This is supported by two lines of evidence. First, we found that exposure to swim stress for 5 or 10 consecutive days did not lead to habituation of the anticonvulsive response even though chronic exposure to stress can lead to adaptations in both behavioral and neuroendocrine responses (Armario, 2006; Johnson et al., 2006; but see Engelmann et al., 2006; Wotjak et al., 1998). These findings may be of particular interest since we previously showed that treatment with the glucocorticoid antagonist mifepristone did not significantly affect convulsion onset times in swim stressed rats suggesting that stress-induced release of corticosteroids are not critical for the anticonvulsant effect of swim stress (Galic et al., 2004). Second, acute restraint stress has been shown to produce higher levels of plasma corticosterone and stress-induced changes in the trafficking of lymphocytes and monocytes compared to a single episode of swim stress (Bowers et al., 2008). Because the magnitude of the hypothalamic–pituitary–adrenal axis response can be considered an indicator of the relative "stressfulness" of an experience (Dallman et al., 1987; Smith and Vale, 2006), one might expect acute restraint stress to exert stronger seizure attenuating effects than swim stress if the apparent "stressfulness" of the experience determines the anticonvulsant efficacy of the stressor. However, we observed that restraint stress was not associated with changes in seizure susceptibility, whereas swim stress significantly decreased seizure susceptibility. Collectively, these results suggest that there is something unique about swim stress over and above the emotional component of "stress" that is responsible for its anticonvulsant effects.

3.2. *Is the protective effect of swim stress related to hypothermia?*

Several studies have found that the behavioral and neurochemical consequences of swim stress depend on the induction of hypothermia (see Bachli et al., 2008; Linthorst et al., 2008; Taltavull et al., 2003). For instance, rapid re-warming in 38 °C water after exposure to cold water prevented reduction in open field exploration in rats (Stone, 1970). Because reductions in core body temperature can suppress the generation of seizure activity in an animal model of electrically induced self-sustaining status epilepticus (Schmitt et al., 2006), we speculated that the protective effect of swim stress against lithium–pilocarpine seizures might be related to the induction of hypothermia after swimming. In support of this idea, we found that swim stress resulted in anticonvulsant effects only when accompanied by a significant (>5 °C) amount of hypothermia after rats swam in water of 20 °C or less; swimming in warm water (35 °C) did not significantly impact convulsion onset times. Remarkably, for lithium–pilocarpine treated rats, the hypothermic condition after exposure to cold water appeared to last well over 12 h before returning to baseline values. Thus, the temperature of water exposure and the induction of sustained hypothermia appear to be critical factors in the emergence of anticonvulsant effects after swim stress.

Recently, Linthorst et al. (2008) demonstrated that lowering water temperature resulted in significantly less immobility or floating and more time spent engaged in swimming or struggling behaviors during a forced swimming task. In other words, rats forced to swim in warm temperatures (35 °C) show less physical exertion (i.e., more time spent floating) than rats forced to swim for a comparable length of time in water temperatures of 25 °C or less. It is possible that differences in overall physical activity might account for the observation that swimming in warm water (35 °C) did not significantly impact convulsion onset time, whereas swimming in water of 20 °C or less resulted in anticonvulsant effects because these conditions promoted greater levels of physical activity. However, it is unlikely that muscular exertion associated with swimming is solely responsible for the anticonvulsant effect of swim stress observed in this study. Although physical activity can reduce the incidence of epileptic seizures for some patients (Nakken, 1999) and decrease susceptibility to lithium–pilocarpine convulsions in rodents (Arida et al., 1999), we found that rats exposed to either a large or restricted swimming environment showed similar convulsion onset times, despite showing different levels of physical activity. Additionally, the size of the swimming environment has also been shown to directly influence the rat's choice of swimming behavior (struggling vs. active swimming vs. immobility) and coping strategy (Detke et al., 1995); however, we did not find that the type of swimming behavior during exposure to restricted or large swimming environments correlated with the convulsion onset time. Further support that the anticonvulsant effect of swim stress may not depend on increased motor activity associated with swimming comes from the observation that with repeated exposures to swim stress, animals will show less motor activity and spend more time engaged in immobile or floating behaviors with each subsequent exposure (Sun and Alkon, 2004). However, we did not find that repeated exposure to swim stress for 5 or 10 consecutive days reduced its anticonvulsant efficacy. While we agree that some minimal level of motor activity is probably necessary for the anticonvulsant effect of swim stress, these findings argue that extreme levels of motor activity or differences in coping strategies during swimming do not appear to be critical parameters for the anticonvulsant effect of this stressor.

The overall pattern of results suggests that the induction of hypothermia that accompanies swimming in cold water appears to be critical for eliciting the anticonvulsant effect of swim stress. The change in body temperature could explain the effects of all of the stressors we tested. For example, even though restraint and nociceptive stress produce mild hypothermic responses (Amar and Sanyal, 1981; Vidal et al.,

1984), the much more pronounced and sustained decreases in body temperature after swim stress would explain why larger anticonvulsant effects are observed after this stressor compared to other acute stressors [Experiment 1, Fig. 1]. When we consider that a duration of at least 5 min of swimming was sufficient to induce an anticonvulsant effect of swim stress [Experiment 2, Fig. 2], that repeated exposures to swimming did not diminish the anticonvulsant effect of this stressor [Experiment 3, Fig. 3], and that the type of behavior engaged in during swimming does not appear to influence the anticonvulsant effect [Experiment 4, Table 1], the most parsimonious explanation is that in each condition a significant change in body temperature always accompanied the swim stress. Importantly, swimming at warmer temperatures (35 °C) did not significantly impact body temperature and was not associated with changes in overall seizure susceptibility, even though swimming at this temperature has been found to increase plasma corticosterone levels compared to non-stressed animals (Drugan et al., 2005). This strengthens our conclusion that the seizure attenuating effects of swimming is dependent upon the induction of hypothermia rather than from anxiogenic or panicogenic properties associated with swim stress. Additionally, administration of drugs that modulate alpha2-adrenergic receptors, which we and others have shown to be important for the anticonvulsant effect of swim stress (Galic et al., 2004; Pericic et al., 2001b), can also promote hypothermic effects in both animals and humans (Bexis and Docherty, 2005; Quail and Shannon, 2003). Finally, our results do not preclude the possibility that changes in brain temperature after swim stress might be the critical parameter mediating the anticonvulsant effect of this stressor. It has been previously reported that brain cooling promotes anticonvulsant effects in both human and experimental animal models of epilepsy (Burton et al., 2005; Gunn et al., 1998; Motamedi et al., 2006; Rothman et al., 2005; Yang et al., 2003). Because rat brain temperature has been shown to vary in parallel with core body temperature (Andersen and Moser, 1995), the rapid induction of hypothermia following swim stress is most likely associated with significant changes in brain temperature. When taken together, our data clearly show that changes in temperature regulation is likely a key determinant in shaping the anticonvulsant outcome of swim stress on lithium–pilocarpine convulsions.

Persistent seizure activity also leads to increased release of glutamate and production of various proinflammatory molecules (such as cytokines) that collectively enhance neuronal excitability and trigger a set of molecular events contributing to progressive neurotoxicity and impaired cell survival (Fujikawa, 2005; Vezzani and Granata, 2005). We found that swimming at 20 °C also provided marked protection against the neurodegenerative effects of lithium–pilocarpine convulsions. These rats had substantially fewer degenerating neurons throughout the hippocampus 24 h after seizure induction compared to non-stressed controls allowed to seize for the same length of time. We believe that this neuroprotective effect might be also related to the strong and long-lasting reduction in body temperature that occurred after treatment with pilocarpine. Although the exact mechanism responsible for this profound and lasting (>12 h) reduction in body temperature is not known, many studies have now reported beneficial effects of hypothermia in diminishing epileptic activity and ameliorating the extent of brain injury after seizures (see Liu et al., 1993; Lundgren et al., 1994; Maeda et al., 1999; Persinger et al., 2001; Schmitt et al., 2006).

3.3. *Considerations*

Because hypothermia can influence the permeability of the blood-brain barrier (BBB) to some compounds, it remains possible that the reduction in seizure susceptibility after swim stress might be explained by changes in the bioavailability of pilocarpine. Several lines of evidence argue against this possibility. Although mild disruptions in the permeability of the BBB in restricted brain regions can occur after stressful manipulations (Theoharides and Konstantinidou, 2007), swim stress appears to increase rather than decrease BBB permeability to tracers, such as Evans blue and ^{131}I-sodium, in rats and mice (Sharma et al., 1991, 1995). Furthermore, a recent study by de Groote and Linthorst (2007) showed that swim stress in rats results in a decrease in extracellular GABA levels in the hippocampus; a region known to possess a low threshold to generate epileptiform activity (de la Prida et al., 2006; Lerner-Natoli et al., 1984). Based on these findings, one might suspect a greater propensity for swim stressed animals to respond to the proconvulsant effects of pilocarpine because: 1) the potential for higher concentrations of pilocarpine in the rat brain resulting from increased BBB permeability after swim stress; and 2) the potential for reduced GABAergic inhibition after swim stress. However, this is clearly not the case. Perhaps most convincing of all is that when rats were exposed to swim stress prior to the administration of pilocarpine, a pronounced and long-lasting reduction in body temperature occurred (Fig. 4). This effect was not observed in rats that were exposed to swim stress but not given pilocarpine (see swim stress only; Fig. 4). Taken together, these findings strongly support the idea that the anticonvulsant effect of swim stress is not simply the result of a decreased propensity to respond to the effects of pilocarpine, but rather a change in the capacity of neuronal circuits to generate seizures when body temperature is reduced.

3.4. *Implications*

The present experiments demonstrate that the swim stress paradigm may be useful for revealing the mechanisms that affect the spread and consequences of seizure activity in the brain. These results further support the idea that the psychological and physiological consequences of stress may not be necessarily the same for all types of seizure disorders explaining why certain stressors can produce proconvulsive or anticonvulsive effects. Further work investigating the mechanism by which body temperature affects epileptic circuitry may help identify additional neurochemical systems that could potentially aid in the treatment of epilepsy.

Our observation that the sustained induction of hypothermia after exposure to swim stress reduces seizure susceptibility suggests that hypothermia may be successful as an adjunct in the management of status epilepticus that is non-responsive to first-line anticonvulsants. There has already been substantial success implementing these procedures for the treatment of

other neurological conditions, such as stroke and traumatic brain injury (Hammer and Krieger, 2003; Shann, 2003). However, the feasibility and safety issues associated with producing hypothermic conditions during the treatment of seizures needs to be considered before wide-spread application of this procedure to clinical situations occurs (Bagic et al., 2008). Finally, our observation that rats forced to swim for as little as 5 to 10 min in room temperature water and still exhibit profound drops in body temperature also raises caution in how such experiments are interpreted in the context of behavioral tests involving stress or learning and memory. With this caveat in mind, we suggest that any such experiments employ body temperature measurements to alleviate such concerns, especially when carried out in association with testing of pharmacological compounds that may potentially influence homeostatic control of body temperature.

4. Experimental procedures

4.1. *Animals*

The subjects were 118 adult male Wistar rats obtained from Charles River Laboratories (QC, Canada) that weighed approximately 300 g at the beginning of the experiment. The rats were housed in pairs (2 to 3 rats per cage) and maintained at a constant temperature of 21±1 °C with food and water available *ad libitum*. They were maintained on a 12:12 h light: dark cycle with lights on at 0700 h.

4.2. *General procedure*

All testing procedures were carried out during the light phase. These procedures were approved by the local animal care committee and were in accordance with the guidelines set by the Canadian Council on Animal Care. All drugs were obtained from Sigma-Aldrich (St. Louis, MO, USA), dissolved in 0.9% physiological saline, and administered at a volume of 1 ml/kg.

The swimming apparatus (dimensions: 200 cm diameter, 60 cm deep) was a standard Morris water maze chamber filled with room temperature water (20 °C±2 °C) to a height of 30 cm, unless otherwise noted. A 10 min duration was used for swim stress exposures to correspond with previous studies (see Galic et al., 2004; Pericic et al., 2000, 2001a), unless otherwise noted. The general procedure was as follows: All subjects were treated with lithium chloride (LiCl; 3 mEq/kg, s.c.) and then returned to their home cages. Four hours later, one group of subjects was exposed to 10 min of swim stress after which they were returned to the previous experimental room for treatment with pilocarpine (30 mg/kg, s.c.). A second group served as non-stressed controls and was handled in a similar manner to the first group except that these rats remained in their home cages during the stress exposure.

Systemic administration of pilocarpine produces a sequence of physical symptoms that is characteristic of increased cholinergic stimulation. These symptoms, which typically begin within 5 to 10 min post-injection, include periods of piloerection, akinesia, salivation, hyperactivity, diarrhea, and chromodacryorrhea. After about 12 min, extensive vibrissae twitching, repetitive head nodding and intermittent periods of myoclonic twitching restricted to the orofacial region can be seen and they resembled the class I and II seizures characteristic of electrical kindling (Racine, 1972). These behavioral changes progress into episodes of motor seizures that would typically begin as unilateral forelimb clonic movements that developed into rearing with bilateral forelimb clonus and loss of postural control, and are analogous to the class V motor seizures first described by Racine (1972). The latency to the occurrence of these latter symptoms was defined as the convulsion onset time. Unless indicated otherwise, all rats were euthanized with an overdose of pentobarbital within 1 h after seizures had commenced.

4.3. *Experiment 1: comparison of swim stress with other acute stressors on seizure susceptibility*

To determine if acute exposure to different physical or psychological stressors can influence susceptibility to lithium–pilocarpine convulsions, a total of 20 rats were assigned to one of the following conditions: 1) thermal nociceptive stress (n=5), 2) 10 min swim stress (n=5), 3) 4 h restraint stress (n=5), or 4) non-stressed controls (n=5).

4.3.1. *Nociception stress group*

Each rat was treated with LiCl and then 4 h later placed on an Omnitech thermal plate (maintained at 55 °C) to elicit nociceptive responses. Once the rat licked one of its hind feet twice in any combination, it was removed from the apparatus and immediately treated with pilocarpine, and the convulsion onset time was recorded.

4.3.2. *Swim stress group*

Each rat was treated as described in the General procedure section above.

4.3.3. *Restraint stress group*

Each rat was exposed to a 4 h restraint stress period immediately after administration of LiCl. The restraint tubes were transparent Plexiglas tubes (25 cm long × 10 cm wide × 6 cm high, VWR, Toronto, ON, Canada) and the length of the restrainer was adjusted to limit the head and limb movements of each rat. Following cessation of the restraint stress, each rat was treated with pilocarpine and the convulsion onset time was recorded.

4.3.4. *Non-stressed group*

Each rat was treated with LiCl and remained in its home cage while the other groups were exposed to the stressors. Four hours later, each rat received a single injection of pilocarpine and the convulsion onset time was recorded.

4.4. *Experiment 2: effect of swimming duration on seizure susceptibility*

To determine if the duration of swim stress influences the anticonvulsant effect of swim stress, a total of 20 rats were treated with LiCl followed 4 h later by an exposure to one of the following swimming durations: 1) 1 min (n=4), 2) 5 min (n=4), 3) 10 min (n=4), 4) 20 min (n=4), or 5) non-stressed controls (n=4). After swim stress, the rats were given pilocarpine and the convulsion onset time was recorded.

4.5. *Experiment 3: effect of repeated presentations of swim stress on seizure susceptibility*

To determine if repeated exposure to swim stress diminishes the efficacy of its anticonvulsant action against lithium–pilocarpine convulsions, a total of 20 rats were exposed to 10 min of swimming with a 24 h interval between successive swim exposures. Rats were exposed to either a single swim stress session ($n=4$) or exposed to 5 days of repeated swim stress ($n=5$) or 10 days of repeated swim stress ($n=5$). A group of 6 rats served as non-stressed controls. On the last day of swim stress, the rats were treated with LiCl, subjected to swim stress 4 h later, and then injected with pilocarpine. The convulsion onset time was recorded.

4.6. *Experiment 4: effect of swim behavior on seizure susceptibility*

To determine if the physical activity inherent in swimming influences the anticonvulsant effect of swim stress, a total of 18 rats were exposed to different sized environments that were designed to elicit different swimming and coping strategies: 1) a large open swim environment that was identical to the apparatus described in the General procedure section ($n=6$), or 2) a restricted swim environment consisting of a narrow Plexiglas cylinder with a height of 30 cm and diameter of 18 cm that was filled to a height of 15 cm with water ($n=6$). A group of 6 rats served as non-stressed controls. A time-sampling procedure was used to identify classical swimming behaviors: swimming (the rat makes active swimming motions), struggling (the rat makes vigorous movements with its forepaws in and out of the water, usually directed towards the walls), and immobility (the rat makes only those movements necessary to keep its head above water). The behavior shown by each rat was recorded every 5 s during the entire 10 min swim exposure for a total of 120 observations. After swimming in the different environments, the rats were treated with pilocarpine and the convulsion onset time was recorded.

4.7. *Experiment 5: effect of water temperature on seizure susceptibility*

We next assessed if the temperature of the water could influence the anticonvulsant effect of swim stress. Under halothane anesthesia (4% induction, 2–2.5% maintenance), 28 rats underwent a standard laparotomy enabling us to surgically implant a sterile miniature silicone-coated temperature datalogger (2 cc, 4.2 g, SubCue, Calgary, AB, Canada) into the peritoneal cavity. A topical anesthetic (4% Xylocaine) was applied along the suture site in order to reduce post-operative discomfort and the rat was returned to its home cage and permitted 4 to 5 days of recovery before experiments commenced. After recovery, the rats were treated with LiCl and 4 h later they were forced to swim at one of the following water temperatures: 10 °C ($n=7$), 20 °C ($n=9$), or 35 °C ($n=8$). A group of 4 rats served as non-stressed controls. After swim stress, 19 of these subjects were administered pilocarpine and the convulsion onset time was recorded. The remaining 9 rats (3 in each temperature condition) were not treated with pilocarpine so that swim-induced changes in body temperature could be studied in isolation. Dataloggers were programmed so that body temperature was measured every 5 min for the duration of testing.

4.8. *Experiment 6: effect of swim stress on seizure-induced neuronal degeneration*

To determine if swim stress protected against the neuropathological consequences of lithium–pilocarpine convulsions, as previous studies have revealed a strong correlation between latency to convulsions and neuronal damage (Persinger et al., 2002), a total of 12 rats were exposed to swim stress at 20 °C ($n=6$) or served as non-stressed controls ($n=6$). After swimming, each rat was given pilocarpine, followed by diazepam (5 mg/kg, s.c., 1 ml/kg) 30 min after the onset of convulsions to standardize the duration of convulsions.

One day later, the rats were given an overdose of pentobarbital and transcardially perfused with cold physiological saline followed by 10% neutral buffered formalin. The brains were removed and postfixed in the same fixative for 48 h at 4 °C. Following postfixation, the brains were blocked and sectioned coronally at 40 μm on a vibrating microtome (Vibratome 3000, Vibratome Company, St. Louis, MO, USA). Sections were stored in a cryoprotectant solution (30% (w/v) sucrose, 1% (w/v) polyvinylpyrrolidone, 30% (v/v) ethylene glycol) at −25 °C until further processing.

Brain sections were washed extensively in 0.1 M phosphate buffer (pH 7.4) in order to remove the cryoprotectant before being mounted on precleaned positively charged glass slides (Superfrost Plus, Fisher Scientific Company, Ottawa, ON, Canada). Mounted tissue was allowed to air dry overnight. Fluoro-Jade B histochemistry was performed using methods described elsewhere (Schmued and Hopkins, 2000). Briefly, sections were rehydrated, pretreated with 0.06% potassium permanganate for 10 min (gently shaken), rinsed in distilled water, and incubated in a 0.001% Fluoro-Jade B (Chemicon, Temecula, CA, USA) and Hoechst 33342 (10 μg/ml, Sigma-Aldrich, St. Louis, MO, USA) working solution for 20 min in the dark at room temperature. Following incubation, the sections were rinsed in distilled water and allowed to dry in a fumehood in the dark before being cleared in xylene and coverslipped with DPX mounting medium (Fluxa, Sigma-Aldrich, St. Louis, MO, USA). Sections were examined using a Nikon E800 epifluorescent microscope with appropriate fluorescent filters and digital images taken using an Optronics camera (Optronics, Goleta, CA, USA) with Microfire software. The number of degenerated cells from 5 sections taken at equal distances between −2.80 mm and −4.52 mm from bregma (Paxinos and Watson, 1998) were manually counted in the dentate polymorphic layer (hilus), Cornu Ammonis (CA) 3 (stratum pyramidale), and CA1 (stratum pyramidale) by an experimenter unfamiliar with the treatment conditions of the animals. To enhance clarity, all digital images were adjusted only for brightness and contrast using Adobe Photoshop CS (Adobe Systems, San Jose, CA, USA).

4.9. *Statistical analyses*

For the majority of analyses, a one-way analysis of variance (ANOVA) was used to investigate between group differences in

convulsion onset time after administration of pilocarpine. Follow-up *post hoc* analyses were done using Dunnett's multiple comparison test where each of the group conditions are evaluated against the control (i.e., non-stressed) values. For Experiment 3, significant group differences were evaluated using a Fisher's protected least significant difference test. For Experiment 4, Student *t*-tests were used to examine if there were differences for each swimming behavior (swimming, struggling, or immobility) during exposure to the restricted or large swim environments. In addition, separate Pearson product moment correlations were calculated for each swimming behavior (swimming, struggling, or immobility) and the convulsion onset time. For Experiment 5, the mean body temperature for each treatment group [10 °C, 20 °C, or 35 °C] was measured at several time points (baseline, 15 min, 0.5 h, 1 h, 3 h, 12 h, 18 h, 24 h) after pilocarpine administration and analyzed using a two-way repeated measures ANOVA. *Post hoc* tests comprised one-way ANOVAs with Fisher's protected least significant differences (to compare group differences in body temperature at each time point) and paired *t*-tests (to compare body temperatures changes after treatment with pilocarpine at different time points for each group separately) with Bonferroni adjustments for multiple comparisons. All statistical analyses were completed using Statistical Package for the Social Sciences v. 13.0 (SPSS, Chicago, IL, USA). Results are expressed as means ± standard deviations (S.D.). The criterion for statistical differences was set at $P<0.05$.

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